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A COMPARISON OF THREE METHODS FOR THE ASSAY OF COMMERCIAL INSULIN PREPARATIONS[1]

BY D. M. YOUNG, D. B. W. REID, AND R. G. ROMANS

Abstract

Each of 14 commercial insulin preparations was assayed for insulin activity using three biological assay methods. In Assay I, 32 rabbits were employed using the "twin cross-over" design. Per cent reductions of blood sugar calculated from blood-sugar levels determined before and one and one-half, three, and five hours after the subcutaneous injection of insulin were used in estimating potency. In Assay II, 16 rabbits were used in a 4 × 4 Latin square design. Blood-sugar levels determined 50 min. after the intravenous injection of insulin were used in estimating potency. In Assay III, 288 mice were employed in a two-level quantal response design. The proportions of animals convulsing in groups of 36 mice after the subcutaneous injection of insulin were used in measuring potency. The agreement between results obtained by the three methods was found to be satisfactory. The average standard error for the estimate of potency was 13% for Assay I; 10% for Assay II; and 9% for Assay III.

The purpose of the present investigation was to compare two rabbit blood-sugar methods and a mouse convulsion method for the determination of insulin activity. Fourteen insulin preparations were assayed by each of the three methods. The estimates of insulin content are presented and some attempt is made to assess the relative efficiency of the different procedures.

Materials and Methods

Each of the insulin preparations used in the study was assayed for its insulin content by the three assay procedures described below. Eleven of the samples were insulin of crystalline or noncrystalline origin, the products of four North American manufacturers; two were samples of Protamine Zinc insulin; and one was a sample of Globin Insulin with Zinc. Some of the samples were from the open market and some were control samples prepared specifically for animal assay.

Assay Method I. Rabbits Receiving Insulin by Subcutaneous Injection

Thirty-two rabbits of mixed breed varying in weight from 1.5 to 3.0 kgm. were used in each assay. The rabbits were fed a commercial ration* and water was available to them at all times. The "twin cross-over" design as used by

[1] *Manuscript received August 2, 1949.*

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

* *Purina Rabbit Chow (Complete ration).*

Smith *et al.* (3) was employed. Each rabbit received one dose of Insulin Standard* and one dose of the unknown under study administered subcutaneously in one ml. of diluting fluid made up to U. S. P. XII (5) specifications. The higher and lower doses of Insulin Standard contained 1.6 and 0.8 International Units respectively. An interval of one week was allowed between the two injections. Samples of blood were drawn from the rabbits at the times and in the manner prescribed by the U. S. P. XII and blood glucose determinations were performed on the individual blood samples by the method of Nelson (2). Per cent reductions of blood sugar, calculated as directed by the U. S. P. XII, were employed in computing the estimates of potency and their standard errors. Reductions in blood sugar of less than 10% were observed in 8% of the animals and these values were arbitrarily excluded from the calculations.

Assay Method II. Rabbits Receiving Insulin by Intravenous Injection

Sixteen rabbits selected and fed as described under Assay Method I were used in each assay. The experimental design employed was that of Bliss and Marks (1), two doses of Insulin Standard (0.6 and 1.2 I.U. per rabbit) and two doses of the unknown sample being administered to each rabbit, one dose on each of four successive working days. The experimental technique was that of Young and Romans (6). Rabbits were starved for 16 to 18 hr. and injected intravenously with the specified quantities of insulin. Each dose of insulin was dissolved in two ml. of diluting fluid made up to U. S. P. XII specifications. Fifty minutes after injection blood was drawn in the customary manner and blood glucose was determined by the method of Nelson. The blood-sugar values so obtained were used directly in computing the estimates of insulin content and the standard errors of these estimates. Duplicate assays were performed on four of the 14 samples used in the study.

Assay Method III. Mice Receiving Insulin by Subcutaneous Injection

One hundred and forty-four female mice were used in each assay. The mice varied in weight from 16 to 24 gm. They were fed on a commercial ration** and water was available to them at all times except during the actual performance of an assay. They were starved for varying intervals of time (4, 6, and 16 hr.) before an assay and they were given at least three days' rest between assays. A two-level assay design of the usual type was employed involving 36 animals per dose. Each dose was administered subcutaneously in 0.25 ml. of U. S. P. XII diluting fluid. The higher dose of both standard and unknown was twice the strength of the lower dose. The experimental technique was patterned after that of Trevan and Boock (4). The mice were injected with preparations containing insulin and placed in groups of 12 in glass-covered copper boxes. The boxes were suspended in a water bath maintained at a temperature of 36.5° to 37.5° C. The numbers of mice

* *Insulin Standard S 230, 23.0 International Units per mgm. (Kindly supplied by the Insulin Committee, University of Toronto.)*

** *Master Fox Breeder Ration (Cube form).*

convulsing within 75 min. of the time of injection were recorded. The estimates of insulin content and the standard errors of these estimates were computed by the probit method. Duplicate assays were performed on all of the samples and the results of these were combined.

Results

In Table I are shown the estimates of insulin content provided by the three methods of assay for each of the 14 insulin preparations used in the study.

TABLE I

ESTIMATES OF INSULIN CONTENT GIVEN BY THREE METHODS OF ASSAY

Insulin sample	Assumed potency I.U./ml.	Estimated insulin content (I.U./ml.)		
		Subcutaneous (32 rabbits)	Intravenous (16 rabbits)	Mouse assay** (288 mice)
1 (Insulin)	40	44.2	39.4	36.2
2 "	20	21.6	18.2	20.3
3 "	20	20.4	23.7	20.0
4 "	20	16.0	17.9	20.8
5 "	40	27.4	27.5 & 27.6*	30.0
6 "	20	21.4	16.4 & 20.4*	24.4
7 "	20	19.5	20.4 & 19.0*	17.3
8 "	20	17.0	20.8	20.0
9 "	20	20.8	17.9	18.2
10 "	20	24.3	19.5	16.1
11 "	20	15.8	23.7	21.2
12 (PZI)	40	38.3	33.4 & 37.4*	36.2
13 (PZI)	40	37.5	44.1	41.6
14 (Globin)	40	39.6	53.0	40.3

NOTE: * *Estimates of potency from two separate assays.*
** *Combined result of two 144-animal mouse assays.*

The table also shows the insulin content assumed for each insulin preparation. Approximate tests of significance, using the standard errors of the individual assays, revealed no real differences among the estimates of potency given by the different methods for any one sample.

The average standard deviation of an individual observation was 7.2% for the subcutaneous rabbit method and 7.9 mgm. % for the intravenous rabbit method. The corresponding average slopes of the response curves were 32.8% and −43.3 mgm. % per log unit. The average slope of the response curves for the mouse method was 4.34 probits per log unit. On the assumption that the assumed potency is not greatly different from the estimated potency, the expected standard error of the estimate of potency would be 13% for a 32-rabbit subcutaneous assay; 10% for a 16-rabbit intravenous assay; and 9% for the combined results of two 144-animal mouse assays. Approximate tests of significance suggested that these standard errors differed significantly from one another.

Conclusions

Commercial preparations of crystalline or noncrystalline insulin, of Protamine Zinc Insulin or of Globin Insulin with Zinc may be as satisfactorily assayed for insulin content by a mouse convulsion method as by either of the rabbit methods employed in this investigation.

Under the conditions described, mouse assays employing a total of 288 mice might be expected to provide more information regarding the potency of a commercial sample of insulin than 16 rabbits used in the intravenous rabbit method, and either of these methods might be expected to provide somewhat more information than 32 rabbits used in the subcutaneous rabbit method.

Acknowledgment

We wish to thank Mr. D. P. Joel, Mr. C. B. Holliwell, and Miss Phyllis Crewe for technical assistance in conducting the assays.

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TOXIN PRODUCTION IN AERATED CULTURES OF *CORYNEBACTERIUM DIPHTHERIAE*[1]

By F. F. Howatt and G. B. Reed

Abstract

In this paper a comparison is made between the growth and toxin production of *Corynebacterium diphtheriae* in aerated and classical still cultures. It is shown that in well aerated cultures fermentation proceeds more rapidly and toxin appears earlier than in still cultures. However, to obtain maximum results in aerated cultures it is necessary to increase the amount of fermentable substance over that present in the media of still cultures. When cultures are aerated with two to three volumes of air per minute and an extra amount of fermentable carbohydrates is added to the growing cultures at a suitable interval a higher yield of toxin is obtained than in still cultures in similar media. The maximum concentration of toxin, moreover, is reached in the aerated cultures after about 60 hr. of incubation as compared with about 120 hr. in still cultures.

In the extensive literature on the production of toxin by *Corynebacterium diphtheriae*, all authors, with one or two notable exceptions, are in agreement that good yields of toxin are obtained only when the organisms grow as a pellicle on shallow layers of suitable fluid media. This opinion is clearly stated in such early papers as those of Roux and Martin (15) and Aronson (2). Twenty-five years later in a general review of the subject Andrewes *et al.* (1) state: "Many workers believe that toxin is only produced when bacteria form a pellicle . . . . . any circumstance which hinders the surface growth of *C. diphtheriae* does not permit the free gaseous exchange which seems so intimately connected with toxin production." Since that time many studies have been made on the ratio of surface exposure to volume of medium, the surface–volume ratio (Pope and Healey (13), Hartley and Hartley (3). Kirkbride *et al.* (7), Pope and Smith (14), Wadsworth and Wheeler (17)). Wheeler and Crowe (18) demonstrated that a reduction in the amount of oxygen in the atmosphere above a surface culture does not seriously affect growth of *C. diphtheriae* but does reduce the yield of toxin.

Siebenmann (16) on the other hand found that the toxin as formed in the pellicle growth tends to concentrate in the surface layer of the culture fluid rather than to diffuse through the mass of the medium. As a result he believes the organisms in the pellicle become partially separated from the nutrients of the medium by the flotation of the toxin and therefore the growth is limited.

With these extensive data on the relationship of toxin production to aeration of the growing *C. diphtheriae*, it is not surprising that Linggood (8) turned to more efficient means of aeration. In a brief paper he has shown that *C. diphtheriae* cultures grown in fluid media aerated by continuous mechanical shaking results in rapid diffuse growth and high yields of toxin. The present

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Contribution from the Department of Bacteriology, Queen's University, Kingston, Ont.

paper is largely a repetition of Linggood's work with added details, particularly in reference to pH changes and concentration of fermentable substances in the aerated cultures.

Experimental Procedure

Medium

Wadsworth and Wheeler's (17) infusion free proteose peptone medium was used throughout this work. Since the results depend to such an extent upon the constitution of the medium the procedure followed in preparing this standard medium is indicated.

The following solution was made from purified salts.

Sodium chloride	5	gm.
Dipotassium phosphate	1	gm.
Monopotassium phosphate	1	gm.
Magnesium sulphate	0.2	gm.
Calcium chloride	0.1	gm.
Peptone-Difco proteose	20	gm.
Distilled water to	1000	ml.

The sodium chloride was dissolved in about one-half the volume of water and the peptone dissolved in this solution. The other salts were dissolved separately in about 50 ml. volumes of water and added to the original solution and the volume made up to 1 liter with distilled water. The pH was adjusted to 7.8 with sodium hydroxide. It was steamed at 100° C. for 30 min., any loss in volume made up with distilled water, and cleared by filtration through soft filter paper.

It was dispensed in 200 ml. amounts in 1 liter pyrex Erlenmeyer flasks. This gives a volume to surface ratio of 0.58, which Wadsworth and Wheeler (17) found to be optimal for their medium. The flasks were loosely plugged with cotton and autoclaved at 115° C. for 30 min.

The following solution was prepared and sterilized by Seitz filtration:

Sodium lactate 70% syrup	36	ml.
Maltose	15	gm.
Glucose	7.5	gm.
Distilled water to	100	ml.

Four ml. of this solution was added to each 200 ml. volume of sterile proteose–salt mixture just before inoculation.

This finished medium, assayed for iron by Hill's (6) method showed, in different batches, from 0.00010 to 0.00012 mgm. of iron per ml. Since this amount was fairly close to the 0.00014 mgm. per ml. shown by Pappenheimer and Johnson (11) to be the optimum concentration for diphtheria toxin production in this type of medium and yield of toxin was sufficient for the comparative purposes of this experiment, no adjustment of the iron concentration was made.

Inoculum

One strain of *C. diphtheriae* was used: Park-Williams No. 8 (Toronto) kindly supplied to us by the Connaught Laboratories. During the course of

this work daily cultures were made in the above described medium in 2 ml. amounts in 1/2 in. test tubes incubated at 35° C. in a nearly horizontal position. Sufficient pellicle was formed in one tube in 24 hr. growth to provide inoculum for 1 to 2 doz. 200 ml. flasks.

All inoculations for either still or aerated cultures were made by floating one loop of pellicle from 24 hr. cultures on the surface of the 200 ml. lots of broth in 1 liter flasks.

Still Cultures

Still cultures were incubated, undisturbed in a well ventilated incubator room at 36° C. Growth was evident in about five hours when small, seemingly isolated, clumps of surface growth could be seen in the vicinity of the original inoculum. After 24 hr. a thin but complete pellicle covered the surface of the medium. This pellicle was not uniform but was dotted with small patches of thicker film. After three days' growth the pellicle was much thickened and wrinkled with festoons of growth descending into the fluid. These festoons appeared to break off subsequently and sink to the bottom of the flasks to produce a heavy sediment. It is apparent that these cultures follow the usual course of toxin producing cultures of *C. diphtheriae* (Pope and Smith (14), Wadsworth and Wheeler (17)).

Aerated Cultures

Aeration was effected by placing the inoculated flasks in a reciprocating shaking machine operated at 90 excursions, of two inches, per minute. This shaking was sufficient to break the surface of the liquid and produce considerable foam but not enough to wet the cotton plugs. Initially, the flasks to be shaken were placed upon the machine immediately after inoculation. This method, however, resulted in total failure of growth in about 20% of the flasks, despite the use of heavier inoculations. To ensure growth in all flasks, a preliminary still incubation of five hours' duration was allowed for all flasks, following which they were placed on the shaker. This five hours' still incubation, which allows some growth of the organism and spread of the pellicle, was sufficient to eliminate growth failures in the agitated cultures.

With this procedure of initial still incubation the aerated cultures rapidly developed a heavy diffuse growth. The medium becomes definitely cloudy in seven to eight hours and in 24 hr. the growth was heavy and evenly diffuse. In the next two to three days the diffuse growth became increasingly heavy with some threadlike clumping of organisms.

Oxidation–Reduction

In still cultures of *C. diphtheriae* grown as just described, the potential at polished platinum electrodes submerged in the media gradually falls from a strongly positive to a negative potential of approximately −200 mv. in 24 to 60 hr. (Fig. 1). This is similar to the potential curve for cultures of this species shown by Hewitt (5). When the culture was continually aerated with 5 ml. of air per minute per 200 ml. of medium the negative drift in potential was less

marked to +50 mv. and with 150 ml. air per minute per 200 ml. of medium the negative shift in potential was still less to +150 mv. (Fig. 1). When electrodes were introduced into cultures subjected to continuous shaking, as

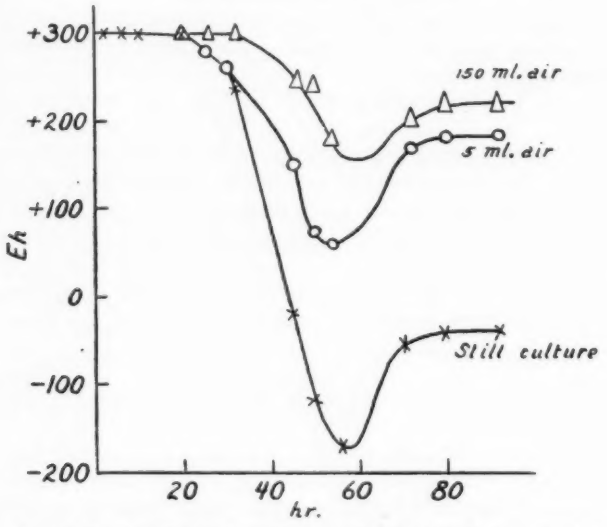

FIG. 1. *Graphs showing changes in oxidation–reduction potentials in growing cultures of C. diphtheriae, lower curve in still culture; middle curve in aerated culture, 5 ml. air per 200 ml. medium per minute; upper curve 150 ml. air per 200 ml. medium per minute.*

described above, it was more difficult to obtain uniform potential readings but the readings fell about midway between those obtained in cultures continually aerated with 5 ml. and 150 ml. air per minute per 200 ml. culture. On this basis it may be estimated that the shaking introduced some 100 ml. of air per minute.

pH and Toxin Determinations

At intervals throughout the period of incubation, 5 ml. samples were drawn off from both the shaken and still cultures. In the case of the latter, great care was taken to produce only minimal disturbance of the pellicle and festoons; the flasks were not handled, and the only disturbance was at the point of entry of the pipette into the medium. These samples were centrifuged for 30 min. at about 3500 r.p.m. This was sufficient to throw down the majority of organisms leaving a clear supernatant. This supernatant, containing the toxin, was decanted into a fresh tube and the sediment was discarded.

The pH of the samples was determined with a glass electrode.

Toxin Assays

Toxin was assayed by the usual Ramon flocculation test and expressed in Lf units. A partially purified, rapid flocculating standard antitoxin was

obtained from the Connaught Laboratory. This antitoxin contained 2000 units per ml. For routine testing it was diluted to 100 units per ml.

Results

Still Cultures

As a preliminary to aeration experiments, toxin production and pH variation in still culture were investigated. Ten numbered flasks were inoculated and placed in the incubator at 35° C. Flasks Nos. 1 to 8 inclusive were sampled at 24-hr. intervals, and the samples were tested for toxin content and pH. Flasks Nos. 9 and 10 were left undisturbed until the end of the experiment to determine whether the entrance of the pipette in sampling caused sufficient disturbance of the pellicle to affect toxin production. The results are shown in Table I and Fig. 2.

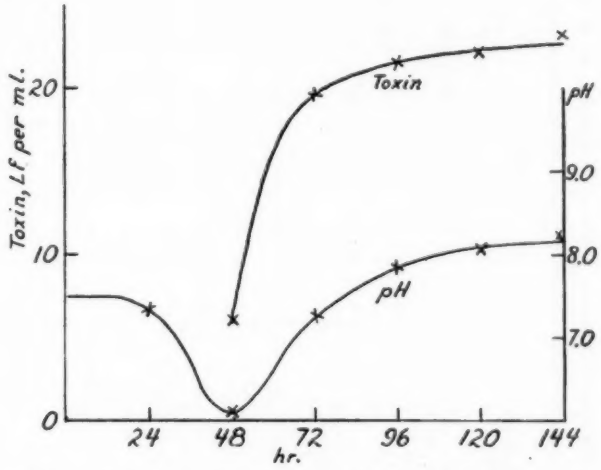

FIG. 2. *Graph showing the average pH change and yield of toxin in Lf units per ml. of a group of still cultures of C. diphtheriae.*

Ordinates represent pH (right), and Lf units of toxin per ml. (left); abscissa time of incubation in hours.

It is apparent that these still cultures followed the usual course of toxin forming *C. diphtheriae* cultures. The pH changes are particularly significant by contrast with aerated cultures discussed in the next section. It is apparent in Table I and Fig. 2 that in these still cultures there is an initial decrease in pH followed by a "reversal" in reaction on the second day and a gradual increase to pH 8.3 on the sixth to seventh day as described by Pope (12) and Andrewes (1). It is well known that the time of "reversal" is related to the surface to volume ratio. With a similar medium and the same surface to volume ratio Wadsworth and Wheeler (17) noted that for high toxin yields the reaction reversal should occur within 72 to 96 hr. and should reach pH 8.0 on the seventh day.

TABLE I

COMPARISON OF THE pH AND TOXIN PRODUCTION, EXPRESSED AS Lf UNITS PER ML., OF 10 SIMILAR *C. diphtheriae* CULTURES

Culture	Time of incubation, hr.																	
	24		48		72		96		120		144		168		192		216	
	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH
1	0	7.4	10	6.1	22	7.4	26	7.8	29	8.0	31	8.1	30	8.3	30	8.4	30	8.3
2	0	7.3	14	5.9	24	7.4	26	7.8	25	8.0	28	8.3	27	8.4				
3	0	7.4	0	6.2	18	7.2	22	8.0	24	8.1	20	8.3	26	8.2	26	8.3	23	8.4
4	0	7.2	10	6.2	17	7.3	23	7.7	24	8.0	26	8.3	27	8.4	27	8.3	27	8.0
5	0	7.3	10	6.1	18	7.2	24	7.8	24	8.1	30	8.1	28	8.3				
6	0	7.5	0	6.2		7.2		7.8	22	8.1	27	8.2	27	8.3				
7	0	7.5	10	6.2	21	7.3	24	7.8	25	8.0	27	8.2						
8	0	7.3	0	6.3	17	7.2	20	7.8	24	8.1	25	8.3	26	8.4	26	8.4		
9																	26	8.4
10				6.15													27	8.3
Av.		7.36	6.7	6.15	19.5	7.27	23.5	7.81	24.6	8.05	26.7	8.22	27.3	8.33	27.2	8.35	26.6	8.28

It will be noted that the daily increase in the amount of toxin present in the samples is greatest in the interval between 48 and 72 hr., that is, immediately after the reaction swings towards the alkaline side. In other words, the greatest daily amount of toxin is produced during an interval when the pH is between 6.0 and 7.2. Further production at higher pH is relatively much smaller, although maximum production is reached only when the pH is definitely on the alkaline side.

Aerated Cultures

An initial series of 10 flasks of the same lot of medium was inoculated from one 24-hr. culture. After a preliminary five hours' still incubation, five flasks were left undisturbed as still controls and five flasks were placed in the shaker and shaken continuously for six days. At intervals, samples were removed from all flasks for toxin and pH assay. The results are shown in Table II and Fig. 3 where the results on five still cultures are averaged and shown in contrast to the average results in five aerated cultures.

TABLE II

COMPARISON OF THE AVERAGE pH AND TOXIN PRODUCTION, EXPRESSED AS Lf UNITS PER ML., OF FIVE STILL CULTURES OF *C. diphtheriae* AND FIVE SIMILAR CULTURES AERATED BY CONTINUOUS SHAKING DURING THE INCUBATION PERIOD

Time, hr.	Still cultures		Aerated cultures	
	pH	Lf/ml.	pH	Lf/ml.
0	7.5	0	7.5	0
5	7.5	0	7.5	0
22	7.4	0	6.7	0
28	7.2	0	7.6	10
34	6.6	0	7.9	20
47	6.1	0	8.3	20
56	6.7	17	8.4	20
72	7.3	20	8.6	19
80	7.5	22	8.7	20
96	7.8	24	8.6	21
116	8.0	25	8.9	20
141	8.2	26	8.8	20

Examination of the curves shows that in agreement with Linggood's (8) results, toxin is formed more rapidly in the aerated than in the still cultures. Reversal of pH occurs earlier in the shaken cultures and in accordance with the findings for still growth, toxin production begins as soon as the reversal reaction occurs. Maximum toxin yield is attained within 36 hr. of inoculation at which time the pH is about 7.9. As incubation proceeds, the pH rises until at 141 hr., it is 8.8 without any further increase in toxin production. This abnormally high pH was noted by Linggood (8), but his report seems to indicate that with his media (papain digest broth and weak tryptic digest broth) the high pH was attained at the same time (four days) as the maximum toxin yield.

The maximum yield with the Wadsworth and Wheeler medium is lower (20 Lf/ml.) in the aerated culture than in the still culture (26 Lf/ml.). It was considered at first that this might be due to oxidation of the toxin as a

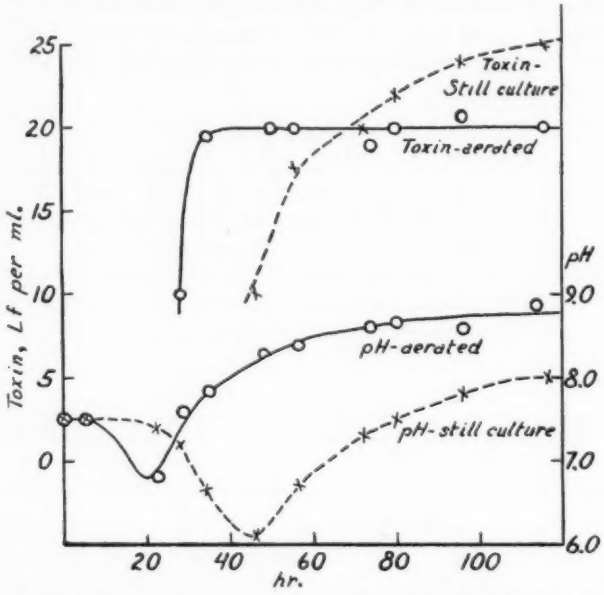

FIG. 3. *Graphs showing the average pH change and toxin yield, expressed as Lf units per ml., of still and aerated cultures of C. diphtheriae.*

Ordinates represent pH (right) and Lf units (left); abscissa, incubation time. Solid line curves represent aerated cultures; dash line curves, still cultures.

result of the increased aeration, but repeat attempts to effect reduction with sodium hydrosulphite according to the method described by Neill (10) failed to increase toxicity.

Aerated Cultures with Additional Carbohydrates

The rapid growth and the early pH changes in the aerated cultures suggested, as a second possibility, that the lower production of toxin might be related to early exhaustion of fermentable substances. Locke and Main (9) found that the addition of 0.1% of glucose to growing still cultures, after reaction reversal, resulted in a definite increase in toxin yield. Helson, Stevenson, and Reed (4) similarly increased the yield of *Clostridium botulinum* toxin by adding a medium concentrate to growing cultures.

A series of 24 flasks of the standard Wadsworth and Wheeler medium with the usual concentration of lactate, maltose, and glucose were inoculated from one 24-hr. culture. They were incubated five hours as still cultures then placed in the shaking machine and shaken continuously for six days. The 24 cultures were divided into four lots of six cultures each. To the first three lots a concentrate of lactate, maltose, and glucose was added in amounts equal

to the initial concentration of these substances. To the first series the concentrate was added after 24 hr. incubation of the culture, or approximately at the point of reaction reversal; to the second series after 28 hr., or some four hours after reversal; to the third series after 36 hr. incubation, or 12 hr. after reversal. The fourth series was left as a control with no added fermentable substances. The first three series of cultures had therefore a total concentration of fermentable substances double that contained in the control cultures.

TABLE III

TOXIN PRODUCTION AND pH CHANGES IN FOUR SETS OF AERATED CULTURES OF *C. diphtheriae*: I—NO ADDITION TO THE GROWING CULTURE; II—ADDITION, AFTER 24 HR., OF CONCENTRATE OF SODIUM LACTATE, MALTOSE, AND DEXTROSE EQUIVALENT TO HALF THE AMOUNT IN THE ORIGINAL MEDIUM; III AND IV—SIMILAR ADDITIONS AFTER 28 HR. AND AFTER 36 HR. INCUBATION

Time	I. No addition		II. Concentrate added 24 hr.		III. Concentrate added 28 hr.		IV. Concentrate added 36 hr.	
	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin
0	7.5	0	7.5	0	7.5	0	7.5	0
5	7.5	0	7.5	0	7.5	0	7.4	0
24	6.8	0	6.8*	0	6.7	0	6.8	0
28	7.6	10	7.0	10	7.5*	10	7.5	10
36	8.0	20	8.0	26	7.8	24	7.9*	20
48	8.3	20	8.1	24	8.2	28	8.2	30
56	8.4		8.2		8.4		8.4	29
72	8.6	19	8.6	23	8.6	28	8.6	30
80	8.7	20	8.4	25	8.6	27	8.9	30
96	8.6	20	8.6	26	8.9	27	8.7	30

* *Indicates time when concentrate of lactate, maltose, and dextrose added.*

Results of the several series are shown in Table III and Fig. 4. It will be noted from the table that in the series to which concentrate was added at 24 hr. the average pH at the time was 6.8 or just beyond the point of reversal of the reaction. In the 28- and 36-hr. series the pH had reached 7.5 and 7.9 at the time the concentrate was added, well beyond the reversal point. The addition of this considerable amount of fermentable material, however, had little influence on the pH of the culture. In Fig. 4 the pH curve is drawn through the observed points in the control cultures without added concentrate. The determined points in the cultures to which concentrate was added fall very close to this curve, Fig. 4.

The yield of toxin, in sharp contrast, is conspicuously increased in the cultures to which the concentrates were added. As indicated in Table III and Fig. 4 the aerated cultures to which no concentrate was added gave a maximum yield of 20 Lf units per ml. after some 40 hr. incubation. In the cultures to which the concentrates were added toxin continued to increase up to 50 to

60 hr. and reached a maximum of 30 Lf per ml. It is also apparent from Fig. 4 that the extent of the influence of added concentrate is related to the interval of the addition. When the addition was made at approximately the

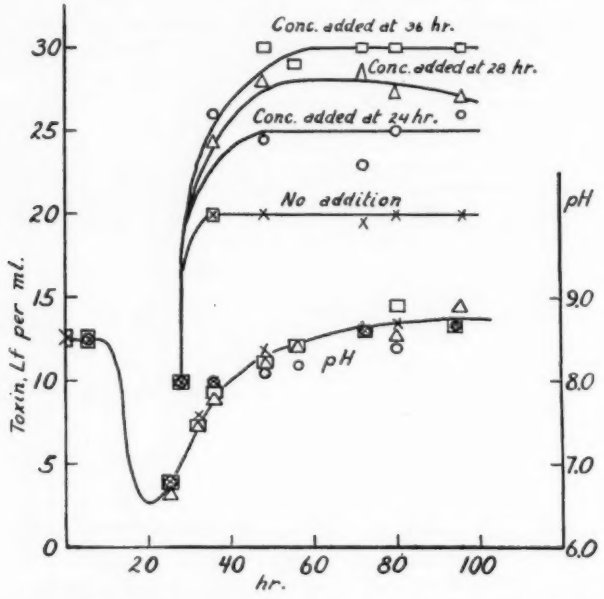

FIG. 4. *Graphs showing average pH and toxin yield in four groups of aerated cultures of C. diphtheriae. The lower curve indicates pH change in all four groups of cultures. The upper curves show toxin yields, successively, in cultures with no addition of fermentable substances beyond that present in the standard medium and with additions of fermentable substances at 24, 28, and 36 hr. after the beginning of the incubation period.*

Ordinates represent pH (right) and Lf units (left); abscissa, incubation time.

point of reaction reversal in the initial culture there was a 25% increase in toxin yield over that in the control culture without addition; when the addition was delayed until some four hours after the reaction reversal there was a 36% increase in the toxin yield and when the addition was delayed to some 12 hr. after reversal there was á 50% increase in toxin yield.

It is apparent from these results that aeration of *C. diphtheriae* cultures markedly alters the metabolism as compared with still cultures. This is evident in the conspicuously different pH curves produced by the two types of cultures, Fig. 3. The difference in metabolism in aerated and still cultures is also evident in the very different oxidation–reduction changes in the two sets of cultures. The most significant difference, however, is the more rapid production of toxin and the higher total yield of toxin, provided an adequate supply of carbohydrates is available, in the aerated cultures as compared with classical still cultures.

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NEUTRALIZING ANTIBODIES AGAINST MOUSE-ADAPTED LANSING STRAIN OF POLIOMYELITIS VIRUS IN THE SERA OF ACUTE AND CONVALESCENT CASES AND NORMAL INDIVIDUALS[1]

By E. L. Barton, N. A. Labzoffsky, W. G. Ross, and L. P. Morrissey

Abstract

The present communication deals with a survey of neutralizing antibodies to mouse-adapted Lansing strain of poliomyelitis virus in the sera of acute, convalescent, and normal individuals during a 1946 epidemic. Two-phase sera were obtained from residents of Ontario and British Columbia and convalescent and normal sera from Quebec. In the sera of 17 out of 35 Ontario patients neutralizing antibodies were demonstrable during the acute stage. In four of these seropositive individuals, there was an increase in the neutralizing titer during convalescence and in three patients there was a notable drop in the titer. The remaining 18 patients were found to be seronegative during both the acute and convalescent stages. Sera from six out of nine British Columbia patients, likewise, contained neutralizing antibodies to the Lansing strain of virus during the acute stage. In four of these the titer remained unchanged during convalescence, in one the titer decreased, and another patient became seronegative. Of the remaining three, two continued to be seronegative and one became seropositive during convalescence. Positive neutralization reactions were obtained with 17 out of 44 convalescent sera from Ontario and 62 out of 146 convalescent sera from Quebec. Sera from 51 children without history of poliomyelitis and 100 adult sera taken at random from specimens submitted for Wassermann tests were obtained from Quebec. Of the children's sera 43%, and of the adults', 48%, contained neutralizing antibodies. The results obtained closely agree with those reported by American workers.

Introduction

Although a considerable amount of work has been done on the neutralization of mouse-adapted Lansing strain of poliomyelitis virus by human sera, the significance of the antibodies remains obscure.

It is generally agreed that neutralizing antibodies against the Lansing strain of virus in poliomyelitic sera appear irregularly (1, 2, 4, 5, 8). In only a very small percentage of clinically recognized cases of poliomyelitis did antibodies appear during the illness, or increase during convalescence (1, 3, 4, 8). In some cases there was a noticeable decrease in antibody titer or complete disappearance of antibodies during convalescence (1, 4, 8). In most instances, however, the antibodies were either absent or, if present, remained apparently unaltered in titer throughout the course of the disease (1, 2, 4, 5, 8).

Further, it has been shown that a considerable number of newborn children contain in their sera neutralizing antibodies against the Lansing virus, presumably as a result of transplacental transmission (6, 7, 9). A very high percentage of normal children, as well as of normal adults, have in their sera neutralizing antibodies to this strain of poliomyelitis virus.

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From the observations made by American workers, it appears that contact with the Lansing or antigenically related strain of virus is widespread, although the percentage of seropositive individuals varies considerably in different geographical areas (1). In view of this it was deemed of interest to determine the incidence of this strain of virus in Canada. For this purpose a limited survey of normal and acute and convalescent poliomyelitic sera from residents of Ontario, Quebec, and British Columbia was made. At the time of this investigation outbreaks of poliomyelitis of epidemic proportions were occurring in the two latter provinces, but there were only sporadic cases in Ontario. The results are reported in this communication.

Material and Methods

Sera

Two-phase sera were obtained from Ontario and British Columbia and convalescent sera were procured from Ontario and Quebec. Normal sera were obtained in Quebec from normal adults whose blood was being subjected to serological tests for syphilis and from otherwise normal children being admitted to hospital as a result of trauma.

The sera were tested for bacterial sterility upon arrival and were kept stored at 4° C. until used. Our own observations and those of others (8, 10) indicate that the neutralizing titer of poliomyelitis serum is not affected by storage at 4° C. for a considerable period of time.

Virus

Mouse-adapted Lansing strain of poliomyelitis virus was obtained through the courtesy of the Bureau of Laboratories, Michigan State Department of Health. After passaging the virus three times in mice, several large groups of animals were inoculated. Spinal cords and brain stems from the paralyzed mice were collected and kept frozen in carbon dioxide ice until a sufficient number were available. A 10% suspension of the pooled cords and brain stems was made in 0.85% sodium chloride solution and then centrifuged for five minutes at 2000 r.p.m. The resulting supernatant fluid was distributed in 1 cc. amounts in ampoules that were sealed and stored in a dry-ice cabinet.

Two such pools were used throughout this investigation. Each pool was tested for sterility and titrated in mice before being employed in the tests. The 50% mortality end point of the pooled material was found to be $10^{-3.5}$.

For the test, one or two ampoules were thawed, the suspension was again centrifuged for five minutes at 2000 r.p.m., and the supernatant diluted 1: 15 with 0.85% sodium chloride, thus yielding a final concentration of virus-infected neural tissue of 1 : 150. This constant virus dilution was used in the test and when mixed with an equal amount of test or normal serum represented approximately 10 LD_{50}.

Neutralization Test

Each serum specimen was tested by mixing an equal volume of undiluted serum and the 1 : 150 constant virus dilution. When comparative titrations

of the sera were made, the following final dilutions of each serum sample: 1 : 2, 1 : 10, 1 : 40, 1 : 160, and 1 : 640 were tested against the above concentration of the virus. In such cases the samples from different bleedings from the same patient were tested simultaneously, allowing the same time of contact between the virus and serum in each mixture. In cases when these titrations revealed a difference in the titer between acute and convalescent serum samples, the particular sera were re-tested using twofold dilutions, the range being determined by the titer found in the first test.

In all cases the mixtures were allowed to stand two hours at 4° C. and one hour at room temperature. Eight mice were inoculated intracerebrally under light anesthesia with each mixture, the dose being 0.03 cc. The sera were tested in groups of 10 to 20, depending on the number of mice available. Known positive and negative serum controls were always included with each test. Inoculated mice were kept under daily observation for paralysis for 21 days. Mice dying during the first 48 hr. after inoculation were not included in the tabulation of the results. If more than one mouse died during the first 48 hr. in any one group of eight, the test was repeated. The test was repeated also if any irregular results occurred in the controls. All mice used were the Swiss strain of albino mice bred in this laboratory, and were 18 to 21 days old.

Interpretation of the Results

The criterion for a positive serum was survival of four or more mice out of seven or eight mice inoculated, and alive after the first 48 hr. The serum was considered negative if three or less survived. The same criterion was used in the titration of positive sera, the end point being taken as the highest dilution protecting 50% or more of the mice.

As a rule, undiluted sera that gave 50% protection in mice were re-tested and were classified as positive only when the results of re-test were in agreement with the first observations. These criteria, which are similar to those of Brown and Francis (1), were adopted in order that our results might be comparable with those of others.

Experimental Data

Acute and Convalescent Sera

The results obtained with 35 two-phase sera from patients in Ontario and nine two-phase sera from patients in British Columbia are summarized in Table I.

From Table I it is seen that 17 out of 35 Ontario patients contained neutralizing antibodies in their sera during the acute stage of illness. In four of these there was fourfold or greater increase in the titer during convalescence and in three there was a significant decrease in the neutralizing titer. The remaining 18 patients of the group were found to be seronegative in the acute as well as the convalescent stage.

TABLE I

SUMMARY OF RESULTS WITH ACUTE AND CONVALESCENT SERA

	Ontario	British Columbia
Total number of patients	35	9
Number positive		
1st bleeding	17	6
2nd bleeding	17	7
3rd bleeding	Not tested	6
Number of paralytic cases in the group	31	8
Number of positives in paralytic group	14	7
Number of nonparalytic cases in the group	4	1
Number of positives in nonparalytic group	3	0
Titer		
Increased	4	1
Decreased	3	2
Unchanged	10	4
Average age		
Seropositive	13.6	9.1
Seronegative	10.2	7.3
For the group	12	8.7
Average time of bleeding		
1st bleeding	5 days	4 days
2nd bleeding	63 days	16 days
3rd bleeding	Not tested	36 days

Six out of nine British Columbia patients were, likewise, found to be positive at the first bleeding. In one of these there was noticeable decrease in the titer when the third bleeding was taken, one patient became seronegative, and in four the titer remained unchanged. Of the remaining three, two continued to be negative but one patient developed antibodies in her blood during convalescence. This patient, a five-year-old girl, was seronegative when tested two days after onset of illness. The second specimen (12 days later) showed, however, antibodies sufficient, in dilution of 1 : 40, to neutralize approximately 10 LD_{50} of virus and the third specimen (40 days after onset) in dilution of 1 : 320.

Convalescent Sera

The results obtained with convalescent sera from Ontario and Quebec are presented in Table II.

Seventeen out of 44 Ontario patients and 62 out of 146 Quebec patients were found to contain neutralizing antibodies in their sera.

In the Ontario group of convalescent sera the incidence of positive reactions was approximately equal among paralytic and nonparalytic cases; in the Quebec group a higher proportion of nonparalytic cases was positive. Whether or not this difference is significant is open to question. However, it must be

TABLE II

SUMMARY OF RESULTS WITH CONVALESCENT AND NORMAL SERA

	Convalescent sera		Normal sera	
			Quebec	
	Ontario	Quebec	Children	Adults
Total number of sera tested	44	146	51	100
Number of positive sera	17 (39%)	62 (42%)	22 (43%)	48 (48%)
Number of paralytic cases in the group	23	99		
Number of positives in paralytic group	9 (39%)	39 (39%)		
Number of nonparalytic cases in the group	21	47		
Number of positives in nonparalytic group	8 (38%)	23 (50%)		
Average age				
Seropositive	10	9.9	7.3	
Seronegative	8.8	6.6	6.1	Adults
For the group	9.3	8	6.6	
Average time of bleeding	72 days	150 days		

pointed out that the Ontario group was small, the average age was somewhat higher, and the interval between onset and bleeding was shorter.

The distribution of seropositive and seronegative individuals for the two sexes was approximately the same in both the Ontario and Quebec groups of convalescent cases.

Normal Sera

Normal children.—Out of a group of 51 sera from children in Quebec, normal with respect to history of poliomyelitis, 22, or 43%, were found to be positive. This percentage of seropositives closely approximates that of the convalescent group, although it must be borne in mind that the normal group was considerably smaller than the convalescent (Table II).

Normal adults.—For comparison, 100 sera from normal adults from the Province of Quebec were included in this study. Unfortunately the age of the donors was not recorded; it was stated, however, that they were adults. Antibodies against the Lansing strain of virus (Table II) were found in 48 out of 100 sera, or 48%.

Discussion

The results presented in this communication on the neutralization of mouse-adapted Lansing strain of poliomyelitis virus by acute, convalescent, and normal human sera agree in general with the findings reported by American

workers. The significant point of this investigation is that further proof is given of the widespread distribution of the Lansing or antigenically related strains of poliomyelitis virus in different geographical areas. The number of sera from Ontario and British Columbia, while large enough to indicate that this virus exists in those areas, is not large enough to provide an estimate of its prevalence.

Findings with the two-phase sera confirm the observations made by others (1, 3, 4, 5, 6, 8) that a considerable percentage of patients contain in their blood stream antibodies against the Lansing strain of virus during the acute stage. The antibody titer in these cases, with a few exceptions, remained the same throughout the illness. In several patients there was noted a considerable decrease in the titer and in one instance a patient who was seropositive during the acute stage later became seronegative. The last phenomenon has been observed by others (1, 8) and offers interesting speculation. Current investigations into the "interference phenomenon" may throw some light on the problem. The case of those patients whose serum contained antibodies in the acute stage of the disease and showed an increased titer during convalescence might be explained in one of two ways: first, that those patients had had a latent infection, which would have given rise to antibodies and later had developed into frank illness; second, that those patients had had a previous infection that had left residual antibodies, but not sufficient protection to prevent reinfection with the same, or an antigenically related, strain of virus. The possibility of such a phenomenon has been suggested by Brown and Francis (1).

The case of the initially seronegative patient who became positive during convalescence can most reasonably be interpreted as a primary infection with Lansing or related virus.

The average age of the positive groups was found to be always higher than that of the negative groups, a fact that has been established by others (1, 7, 8, 9). The proportion of positive sera was approximately the same in convalescent children as in normal children and adults.

It would appear that the Lansing strain of virus alone was not the cause of these outbreaks. The high percentage of seropositive individuals, on the other hand, suggests that there had been a recent and rather widespread contact with the Lansing or antigenically related strain in these three provinces of Canada.

Acknowledgment

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THE RELATIONSHIP OF DIETARY FACTORS TO RAT SERUM ALKALINE PHOSPHATASE

1. THE EFFECT OF FAT, METHIONINE, AND CYSTINE[1]

By Jules Tuba and Ridley K. Shaw

Abstract

In synthetic diets fed to weanling rats, methionine and fat must be present in a definite ratio in order to maintain a serum alkaline phosphatase activity equal to that obtained on a standard laboratory diet of animal checkers. This ratio is approximately 1 : 25 by weight for a diet containing 8.5% fat. Increased fat enhances, while increased methionine lowers, the serum phosphatase activity. Although in some experiments methionine was fed in concentrations sufficient to lower phosphatase activity to what has been considered definitely subnormal values, growth was good and the general condition of the animals was excellent. However, beyond certain concentrations of the amino acid, food consumption decreased and weight losses occurred. Cystine had no effect in opposing the action of methionine on serum alkaline phosphatase.

Introduction

There is extensive evidence in the literature that the concentration of alkaline phosphatase in rat serum may be rapidly and significantly altered by a number of dietary factors or the nutritional state of the animal. Weil and Russel (13) found that starvation decreased plasma phosphatase activity and that these lowered levels were elevated following the ingestion of certain unsaturated fatty acids, while saturated fatty acids, proteins, and carbohydrates had no effect. Hough *et al.* (6, 7) observed a pronounced rise in the serum phosphatase concentration of dogs that received a diet high in fat and low in protein. Their work indicated that the level of the enzyme varied directly with the concentration in the diet of labile methyl groups in the form of protein, methionine, or choline. They found that cystine increased the serum alkaline phosphatase activity and attribute to cystine an antilipotropic effect, as do Salmon (8) and Treadwell (10).*

Considerable work has already been reported from this laboratory concerning the effect on serum alkaline phosphatase of a variety of experimental conditions. Tuba, Baker, and Cantor (11) found a pronounced diurnal variation in the level of the enzyme, associated with the time of feeding, which necessitated bleeding the animals at 8 a.m. in order to standardize the effect of this factor. They noted further that the enzyme concentration varied directly with the total daily consumption of the stock laboratory diet. It is evident from their results that the influence of sex, castration, and sex hormones upon the enzyme activity can be directly correlated with alteration in the quantity of food ingested daily. Cantor, Wight, and Tuba (2) presented further evidence

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* *This confirms the original observation of A. W. Beeston and H. J. Channon. Biochem. J. 30 : 280. 1936.*

relating food intake to serum phosphatase activity, as well as proof that it is the fat content of the diet rather than the caloric intake that determines the level of the enzyme. Tuba, Cantor, and Richards (12) fed to weanling rats a diet consisting chiefly of barley with a low protein content, which elevated the serum alkaline phosphatase values about 75% above that obtained on a stock laboratory diet. The addition of methionine to the low protein barley diet resulted in very nearly normal concentrations of the enzyme.

The work reported here is an attempt to elucidate the relationships between serum alkaline phosphatase and dietary fat, methionine, and cystine.

Experimental

Immature, male albino rats were caged individually and given food and water ad libitum. Each diet was fed to a group of 10 animals if possible for a period usually of six weeks. The rate of growth and food consumption of each animal was noted.

Bleeding for estimation of serum alkaline phosphatase concentration was from the tail. Serum was stored at 5° C., when necessary, with unaltered phosphatase activity but enzyme levels were determined within two to three days after the blood was collected. Enzyme values were obtained for each group at intervals during the feeding period. Serum phosphatase was determined by the micromethod of Shinowara, Jones, and Reinhart (9), as modified by Gould and Schwachman (5). The unit of phosphatase activity is defined by Shinowara as "equivalent to one milligram of phosphorus liberated as phosphate ion during one hour of incubation at 37° C., with a substrate containing sodium β-glycerophosphate, hydrolysis not exceeding 10% of the substrate and optimum pH of the reaction mixture for the alkaline enzyme at 9.3 ± 0.15."

The diets all contained 4% McCollum's Salt Mixture, 2% cod liver oil, 0.1% choline chloride, and adequate supplements of thiamine, pyridoxine, pantothenic acid, nicotinic acid, and riboflavin (12). The remaining 94% was made up with sucrose, Crisco, and vitamin-free casein in the proportions indicated below for each diet. Protein was maintained at levels no higher than 10% because experiments to be published in a subsequent paper indicate that 10% protein would permit a steady though subnormal rate of growth, and at the same time this low value of protein would keep at a minimum the lipotropic action of this dietary factor.

Results

The Effect of Varying the Dietary Fat Concentration

Weanling male rats, 21 days old, were placed on diets containing 10% protein, vitamin and salt supplements, fat as indicated in Table I, and sucrose in amounts required to complete each diet. Groups of 10 animals were maintained on each diet for six weeks and the average daily consumption, as well

as the average alkaline phosphatase values for each group, are given at two-week intervals. The standard deviations are indicated for the terminal phosphatase values. The growth rates were almost identical and are not included.

TABLE I

THE EFFECT OF DIETARY FAT CONCENTRATION ON AVERAGE SERUM ALKALINE PHOSPHATASE LEVELS (UNITS PER 100 ML.) (P) AND AVERAGE DAILY FOOD CONSUMPTION (GM. PER DAY) (C) OF WEANLING RATS

Diet No.	Fat in diet, %	2 weeks		4 weeks		6 weeks	
		P	C	P	C	P	C
1	25.0	252	4.7	244	5.3	237 ± 41*	5.9
2	8.5	98	5.9	103	6.2	99 ± 12	7.0
3	5.0	74	5.6	95	9.1	82 ± 13	8.9

* *Standard deviations are indicated for phosphatase values at end of experiment.*

The results in the above table show clearly that increased fat concentration enhanced the serum alkaline phosphatase activity, although at the same time the daily consumption of food tended to decrease. This is in complete agreement with previous work in this laboratory, although Weil and Russel were able to obtain such parallelism only up to a dietary concentration of 8% lard.

THE EFFECT OF METHIONINE

Because both methionine and fat have been shown to influence serum alkaline phosphatase levels, it seemed very likely that the ratio of these two in the diet (M : F) might be of critical importance in determining the concentration of the enzyme. In the work reported previously from this laboratory on the effect of methionine (12) the ratio of the total amino acid (in protein and supplement) to fat was 1 : 70 and this produced an abnormally high enzyme activity. Diet 1 above was designed to have a ratio of M : F* = 1 : 70, and again very high phosphatase values resulted. In Diet 2 the M : F ratio was 1 : 25 and this effected a level of enzyme activity approximately equal to that obtained with the stock laboratory diet of fox checkers. The M : F ratio in Diet 3 was 1 : 14 and reduced the enzyme activity to subnormal values.

The following two diets were designed to further clarify this apparent relationship between these dietary factors and the serum enzyme. The rates of growth were similar to those for the three diets described above.

Diet 4

This contained 5% fat and a supplement of 0.35% DL-methionine, which, taking into consideration the methionine content of the 10% casein, resulted in a ratio for M : F of 1 : 7. A group of eight weanling rats was fed this diet

* *The methionine content of casein was based on values given in Block and Bolling (1).*

for the usual six-week period. The increased M : F ratio produced by the fifth week a very pronounced lowering of the enzyme activity, which remained at this level until the termination of the experiment. In Table II this is compared with Diet 2 (M : F = 1 : 25), which also contained 5% fat and produced "normal" enzyme activity. The consumption was slightly greater

TABLE II

THE EFFECT OF INCREASING THE METHIONINE TO FAT RATIO (M : F) ON AVERAGE SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P) AND AVERAGE FOOD CONSUMPTION (GM. PER DAY) (C)

Diet No.	M : F ratio	2 weeks		4 weeks		5 weeks		6 weeks	
		P	C	P	C	P	C	P	C
2	1 : 25	98	5.9	103	6.2	92	6.4	99 ± 12*	7.0
4	1 : 7	98	7.7	90	8.7	50	7.7	45 ± 10	7.7

* *Standard deviations are indicated for phosphatase values at end of experiment.*

with Diet 4 than with Diet 2, and this ordinarily should have increased the phosphatase activity. The rate of growth of the animals was normal and they appeared to be in good health although such low phosphatase levels on the standard diet of fox checkers would have indicated some abnormal condition.

Diet Five

A group of 10 weanling rats was fed a diet containing 25% fat and 10% protein (M : F = 1 : 70) for three weeks, and then 1.45% methionine was added as a supplement (M : F = 1 : 14). A very high enzyme concentration produced by three weeks on the high-fat, low-methionine diet was decreased 45% in 14 days by the methionine supplement, as indicated in Table III. However, the high fat concentration counteracted the methionine effect to such an extent that enzyme activity was not restored to normal, much less to the

TABLE III

THE EFFECT OF A METHIONINE TO FAT RATIO OF 1 : 14 IN RELATION TO VARIATION OF THE FAT CONTENT OF THE DIET ON AVERAGE SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P) AND AVERAGE FOOD CONSUMPTION (GM. PER DAY) (C)

Diet No.	Fat, %	0 days		2 days		7 days		14 days	
		P	C	P	C	P	C	P	C
3	5	—	—	—	—	—	—	74 ± 14*	5.6
5	25	296	6.0	195	4.4	171	4.6	167 ± 35	5.0

* *Standard deviations are indicated for phosphatase values at end of experiment.*

subnormal values obtained with Diet 3, which also had M : F = 1 : 14, but contained only 5% fat. A diet containing M : F = 1.7 was fed to these same animals, but this methionine supplement of 3.6% resulted in a loss of weight. This experiment was discontinued because the loss in weight, which always results in decreased serum alkaline phosphatase activity, would inevitably have masked the effect of the methionine itself.

The Effect of Cystine

The methionine-opposing action of cystine is said to be most marked on a high-fat, low-protein diet. Treadwell (10) has indicated that such a ration will produce fatty livers in rats. It was shown with Diet 5 above that weanling rats subjected to this regime would in three weeks manifest serum alkaline phosphatase levels as much as 200% above normal. Methionine supplements introduced at the end of the three-week lipogenic period had a potent influence in diminishing phosphatase activity. It seemed probable that counter effects of cystine and methionine on the enzyme might also be best demonstrated when concentrations in serum were at high levels.

Diets 6 and 7

Twenty weanling rats were fed a basal diet of 10% casein and 25% fat for three weeks. Growth curves were the same as previously obtained with this diet. A supplement of 1.8% L-cystine was then fed to a group of 10 of the animals (Diet 6) and the remaining 10 received supplements of 1.8% L-cystine and 1.45% DL-methionine (Diet 7). Consumption and serum phosphatase activities are recorded in Table IV at the termination of the three-week period on the basal diet and at intervals for three weeks while the amino acids were being fed. The supplementary cystine did not in this three-week interval

TABLE IV

THE EFFECT OF SUPPLEMENTARY CYSTINE AND METHIONINE ON AVERAGE SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P) AND AVERAGE FOOD CONSUMPTION (GM. PER DAY) (C) OF RATS PREVIOUSLY FED A DIET CONTAINING 25% FAT AND 10% PROTEIN

Diet No.	Supplement	0 days		2 days		1 week		2 weeks		3 weeks	
		P	C	P	C	P	C	P	C	P	C
6	1.8% cystine	235	6.0	254	7.4	235	7.0	206	7.6	198 ± 24*	9.5
7	1.8% cystine, 1.45% methionine	250	7.4	167	4.6	174	6.6	161	6.6	165 ± 27	7.4

* *Standard deviations are indicated for phosphatase values at end of experiment.*

produce an enhancement of enzyme activity, as reported by Hough *et al.* (7) on a high-fat, low-protein, low-choline diet. In fact, a slight decrease occurred in phosphatase levels below the values obtained with Diet 1. Although not shown in Table IV, Diet 6 was fed for five weeks by which time the animals

lost weight quickly, enzyme activity decreased, and the animals began to die. Curtis and Newburgh (3, 4) showed that if cystine were fed in amounts exceeding growth requirements death would eventually result. Diet 7 resulted in phosphatase levels identical with those obtained with Diet 5. There was with this diet no demonstrable opposition between cystine and methionine with regard to serum alkaline phosphatase activity.

A choline-free diet, otherwise identical with Diet 6, was used in a further experiment similar to the above two. The addition of cystine to this diet resulted in an improved nutritive state: food consumption and growth rate increased, and phosphatase levels increased but not beyond those obtained with Diet 6. Paired-feeding experiments with the choline-free diet also failed to show any cystine effect.

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STEROID EXCRETION OF INFANTS[1]

By W. F. Perry and H. Chochinov

Abstract

The urinary excretion of corticosteroids and ketosteroids was measured in infants during the first week of life and at four and 18 months of age. It was found that corticosteroid excretion increased with age while the excretion of ketosteroids declined from birth, but was again at a similar level to that of one- to three-day-old infants in the 18-months-old child. The excretion of androgenic substances did not parallel the excretion of ketosteroids, these substances being present in minute amounts at birth and rising gradually with increasing age. It is suggested that the involution of the fetal adrenal cortex is associated with a declining production of nonandrogenic ketosteroids.

Introduction

The adrenal cortex of infants is of interest in that it is relatively larger than that of adults and, in addition to the definitive or adult type of cortical cells, there is present a wide zone of cells variously known as the fetal cortex, the "androgenic" zone or the "X" zone. This zone undergoes involution rapidly during the first two weeks postpartum and then more slowly until, by the end of the first year of life, the process is complete. As the significance of this change is obscure it was decided to determine whether it was associated with alterations in the excretion of ketosteroids and corticosteroids, i.e., substances of adrenal cortical origin.

Subjects and Methods

The subjects were essentially healthy and consisted of 24 male infants between one and seven days old, a two months premature female infant, and two male infants aged four and 18 months. It was found impossible to make accurate and complete 24 hr. collections from these subjects, so it was decided to measure excretions as amounts per liter of urine rather than as amounts per 24 hr. In order to obtain sufficient quantities of urine for assay of androgenic activity the subjects between the ages of one and seven days were divided into two groups made up respectively of 15 infants between the ages of one and three days and nine infants between the ages of four and seven days. Four of the infants were in both groups at the appropriate time after birth. The urine of the members of each group was pooled, no one individual contributing more than 20% of each pool. No preservative was used but the varying amounts of urine collected each day from each child or group of children was immediately frozen and kept so until the various determinations were performed. Approximately 1800 ml. of urine was collected from each group of children; in the case of the premature and the two older infants a similar amount was collected from each child.

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Contribution from the Department of Physiology and Medical Research of the University of Manitoba, and from the Winnipeg General Hospital, Winnipeg, Canada.

Ketosteroids were determined by means of their characteristic reaction with *m*-dinitrobenzene in an alkaline medium to form a red pigment. The corticosteroids were determined using principles of the methods of Heard and Sobel (3) and Talbot *et al.* (7). The procedure (6) is essentially an estimation of the reducing power of the neutral lipid soluble substances of urine and for normal adults gives values between 0.5 and 0.9 mgm. per 24 hr. The test for androgenic activity was carried out after the method of Frank *et al.* (2). This test is based on the growth of chick combs following daily topical application, for seven days, of the test substance dissolved in alcohol, androsterone being used as a reference standard. The urine extracts were prepared by acid hydrolysis of 1500 ml. of urine followed by extraction with carbon tetrachloride. The carbon tetrachloride was washed with alkali and water and then evaporated to dryness. The dry residue was taken up in 2 ml. of ethanol and 0.05 ml. was applied daily to each chick. Four to five chicks were used for each unknown and for each of the doses of the standard.

Results

Although accurate daily outputs of urine were not obtained, one may approximate the daily steroid output by taking the average daily urine volumes to be 70 ml. for the one- to three-day-old infants, 140 ml. for the four- to seven-day-olds, 300 ml. for the four-months-old infant, and 500 ml. for the 18 months child. In Table I the observed concentrations of steroids and the calculated outputs are recorded. It will be seen that the excretion of corti-

TABLE I

STEROID CONTENT OF INFANTS' URINE

Subjects	Corticosteroids		Ketosteroids		Androgenic activity as androsterone		
	Mgm./liter	Mgm./24 hr. (calculated)	Mgm./liter	Mgm./24 hr. (calculated)	μgm./liter	μgm./24 hr. (calculated)	% total ketosteroids
Premature	0.47	—	7.0	—	0	—	0
1 to 3 days	1.01	0.07	16.0	1.1	18	1	0.1
4 to 7 days	0.43	0.06	6.5	0.9	18	2	0.3
4 months	0.55	0.17	1.6	0.5	32	10	2.0
18 months	0.56	0.28	2.7	1.3	120	60	5.0

costeroids increased after the first week while the ketosteroid excretion declined from birth to four months and increased again in the case of the oldest infant. The androgenic activity did not parallel the ketosteroid excretion but showed a continuous increase with age. As well as an absolute increase in androgenic excretion the percentage of the total ketosteroids with androgenic activity also increased with age.

Discussion

Ketosteroids and corticosteroids were excreted in higher concentrations by infants one- to three-days-old than by those who were older. This suggests a

postpartum excretion of substances of maternal origin but the very low androgenic potency of the ketosteroids excreted at that time does not support this view. It is more likely that the change in concentration is a reflection of the change in water excretion.

The concentration of corticosteroids in infants' urine is similar to that of adults, though the daily excretion is much lower. Matson and Longwell (4) noted that the excretion of lipid soluble reducing substances by infants was much lower than by adults and considered that the amounts excreted had no correlation with the age or prematurity of the child. However, if the daily excretion were calculated on a body weight basis it appeared that the excretion of these substances was greater in infants than in adults and they suggested that this greater excretion might be due to loss of stored materials occasioned by the involution of the cortex. The progressive increase with age found in the present series does not support this view.

Day (1) found the ketosteroid excretion of infants between the ages of one and five days to be from 0.1 to 3.4 mgm., averaging 0.9 mgm., similar values to those reported here. More recently Venning (8) has explored in detail the changes in ketosteroid excretion during the first nine days of life. In three infants she has found excretions between 1.5 and 1.8 mgm. during the first day with a progressive fall each day thereafter. This, too, is in conformity with the present findings.

Only a small proportion of the ketosteroid material of the infants was found to have androgenic activity but this proportion increased with age. This is contrary to a recent report (5) that there are no androgens present in the urine of children until the age of eight years. However, Wood and Gray (9) on the basis of the difference in values obtained by estimating ketosteroids by the Zimmerman and antimony trichloride reagents, the difference representing the amount of dehydroisoandrosterone, conclude that children between the ages of two and eight years excrete relatively more of this poorly androgenic steroid than do adults and further that the younger children excrete relatively more than do older children. Dehydroisoandrosterone is considered to be about 1/10th as active as androsterone. If all the ketosteroid material in the infants' urine were this substance the biological activity in the case of the one- to three-day infants, for example, would be equivalent to 1.6 mgm per liter, whereas it was only 18 μgm. per liter. In other words the majority of the ketosteroids in infants' urine are androgenically inert, though the proportion increases with age.

From the evidence presented here it is suggested that the involution of the "X" or "androgenic" zone of the infant adrenal cortex is accompanied by a declining production of nonandrogenic ketosteroids. The growth of the adult type of cortex as the infant ages is associated with gradual increase in output of corticosteroids and of ketosteroids with androgenic activity.

Acknowledgment

We wish to thank Dr. H. Medovy, Pediatrician to the Winnipeg General Hospital, for his interest and help in this investigation. The work was done while H.C. was a Scholar of the Manitoba Institute for the Advancement of Medical Education and Research.

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A NOTE ON THE EFFECT OF AN INTRAVENOUS INJECTION OF BILE SALT ON THE PLASMA PHOSPHATASES OF THE RABBIT[1]

By W. F. Haight

Abstract

Since it has been established (*a*) that the alkaline phosphatase activity of the plasma is increased in obstructive jaundice, (*b*) that the white cells are rich in alkaline phosphatase, (*c*) that this alkaline phosphatase is liberated from the cells in the presence of bile salts, and (*d*) that bile salts accumulate in the blood when the biliary tract is obstructed, there remained the possibility that the increase in plasma alkaline phosphatase observed after obstruction to the biliary tract might be the result of the liberation of alkaline phosphatase from the white cells by the retained bile salt. However the injection of bile salt into the blood stream of rabbits caused an increase in the acid phosphatase activity of the plasma with no significant change in the concentration of alkaline phosphatase. Since there is no increase in the acid phosphatase of the plasma in obstructive jaundice, these experiments provide no evidence for the theory that the increase in plasma alkaline phosphatase that follows biliary obstruction is the result of the retention of bile salt.

Introduction

Previous work in this laboratory has shown that white cells are rich in alkaline phosphatase (9) and that this alkaline phosphatase is liberated from the white cells by the *in vitro* action of surface-active substances, such as bile salt, saponin, or alkyl sulphate (22). It has frequently been suggested that, if this mechanism were active *in vivo*, it could explain the high plasma alkaline phosphatase values observed after obstruction to the biliary tract, since in this condition surface-active bile salt is retained in the blood. A high concentration of bile might liberate alkaline phosphatase from the white cells of the peripheral blood or from those of other organs rich in white cells, e.g. spleen or bone marrow. Such an explanation of the increase in plasma alkaline phosphatase observed in obstructive jaundice would amount to a revival, in a modified form, of the 'activator' theory of Thannhauser, Reishel, Gratton, and Maddock (27).

This suggestion has been put to the direct test by injecting bile salts into the blood stream of rabbits and observing the changes in both the acid (pH 4.9) and the alkaline (pH 9.9) phosphatase of the plasma.

Methods

Either sodium desoxycholate or sodium taurocholate, in the quantities and concentrations shown in Tables I and II, was injected into the marginal ear vein of a rabbit. Thirty minutes later blood was withdrawn from the vein of the opposite ear for the determination of the plasma phosphatases. The acid phosphatase was determined by the method of Watkinson, Delory, King, and Haddow (29) and the alkaline phosphatase determined by the method of King, Haslewood, Delory, and Beall (15).

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Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont. The research was supported by a grant from the National Research Council of Canada.

Results

Table I shows that 30 min. after the injection of 125 mgm. of sodium desoxycholate there was a great (565%) increase in the activity of the acid phophatase of the plasma with only a negligible (19%) increase in the activity

TABLE I

THE EFFECT OF INTRAVENOUS SODIUM DESOXYCHOLATE ON THE SERUM ACID AND ALKALINE PHOSPHATASE OF THE RABBIT

Volume injected, ml.	Conc. of sodium desoxycholate, %	Total quantity injected, mgm.	Acid phosphatase activity, King–Armstrong units/100 ml.			Alkaline phosphatase activity, King–Armstrong units/100 ml.		
			Before	30 min. after	Percentage increase	Before	30 min. after	Percentage increase
5	2.5	125	7.71	51.30	565	3.31	3.93	19
4	2.5	100	16.50	53.10	222	4.84	5.66	17
3	2.5	75	8.88	11.91	34	2.87	3.51	22
5	0.5	25	16.90	15.45	−3	4.36	4.67	7

of the plasma alkaline phosphatase. Lesser quantities of sodium desoxycholate (100 mgm. and 75 mgm.) produced a similar, though smaller, increase in acid phosphatase activity. When the quantity injected was reduced to 25 mgm., there was no significant increase in the activity of either phosphatase.

Table II shows that sodium taurocholate had an action similar to that of sodium desoxycholate, except that the quantity necessary to produce the same effect was greater. Injection of 1000 mgm. of sodium taurocholate produced

TABLE II

THE EFFECT OF INTRAVENOUS SODIUM TAUROCHOLATE ON THE SERUM ACID AND ALKALINE PHOSPHATASE OF THE RABBIT

Volume injected, ml.	Conc. of sodium taurocholate, %	Total quantity injected, mgm.	Acid phosphatase activity, King–Armstrong units/100 ml.			Alkaline phosphatase activity, King–Armstrong units/100 ml.		
			Before	30 min. after	Percentage increase	Before	30 min. after	Percentage increase
10	10	1000	3.27	57.30	1652	5.12	8.16	59
5	10	500	7.02	30.00	327	2.19	2.27	4
5	10	500	12.90	33.60	160	5.69	6.62	16
1	10	100	9.75	10.38	6	4.79	4.56	5

a great (1655%) increase in the acid phosphatase activity of the plasma and a very much smaller (60%) increase in the activity of the alkaline phosphatase. The injection of 500 mgm. sodium taurocholate produced a considerable increase in the activity of the acid phosphatase, but no great change

in activity of the alkaline phosphatase. When the amount injected was reduced to 100 mgm., the sodium taurocholate was without effect.

In the experiments presented in Tables I and II the phosphatase activity was measured 30 min. after the injection of the bile salt. Fig. 1 shows that the plasma acid phosphatase activity slowly decreased from this time and that 24 hr. after the injection both the acid and the alkaline phosphatase activity had fallen to within the normal range.

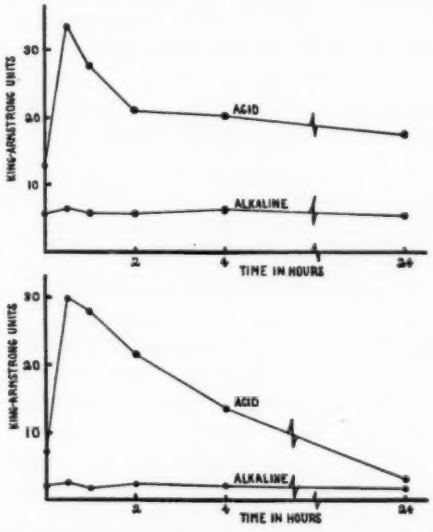

FIG. 1. *Change in the serum phosphatase levels of rabbits following the intravenous injection of 5 ml. of 10% sodium taurocholate.*

Discussion

This work was undertaken in the hope that it might lead to an understanding of the mechanism of the increased alkaline phosphatase that undoubtedly occurs in the serum of both man (reviewed in 14, 18, 19) and experimental animals (5, 11, 24) after obstruction to the biliary tract. In this condition there is also a retention of surface-active bile salt in both man (23, 25) and experimental animals (6, 12, 17, 23). Since it has been shown that alkaline phosphatase is liberated from the white cell in the presence of bile salt *in vitro* (22) and also that the white cells of peripheral blood and other organs rich in white cells contain large amounts of alkaline phosphatase (9), it was possible that the retained bile salt might cause the increased alkaline phosphatase of obstructive jaundice by liberating the enzyme from the cells *in vivo*. However it was found that injection of bile salt into the blood stream of a rabbit in amounts far too small to produce a significant change in the plasma alkaline phosphatase activity produced an increase in the acid phosphatase activity. Since there is no increase in acid phosphatase in the sera of animals with biliary

obstruction (8), it is concluded that there is not sufficient retention of bile salt to explain the increase in alkaline phosphatase activity. The amount of bile salt that causes a rise in plasma acid phosphatase activity is much less than that necessary to produce a rise in alkaline phosphatase activity.

The acid phosphatase that appears in the plasma is possibly derived from the red cells. Red cells are known to be rich in phosphatase active at pH 4.5 to 5.5 (4, 16) and bile salt is known to lyse red cells. After an injection of bile salt the serum always showed evidence of some haemolysis. Lysis of 10% of the red cells would be sufficient to increase the plasma acid phosphatase some 20 King–Armstrong units per 100 ml., a rise of the same order as that found after the injection of bile salt.

Four theories have been advanced to explain the increase in serum alkaline phosphatase that occurs after biliary obstruction:

1. The liver produces more alkaline phosphatase than normal. Evidence for the hepatic origin of serum phosphatase has been put forward by Bodansky and Jaffe (5), Greene, Shattuck, and Kaplowitz (13), Freeman, Chen, and Ivy (11), and Oppenheimer and Flock (21).

2. The liver cannot excrete the normal amount of alkaline phosphatase and hence there is a retention of the enzyme in the blood. This view is held by Armstrong and King (2, 3) and evidence supporting the theory has been summarized by Gutman *et al.* (14). Both the histochemical studies of Wachstein and Zak (28) and the careful correlation of chemical and histochemical methods by Sherlock and Walshe (26) favor the retention theory.

3. The absence of bile in the intestine interferes with fat and calcium absorption. This produces an osteoporosis with a high serum alkaline phosphatase (Morris and Peden (20)). As Sherlock and Walshe (26) point out, this view would appear unlikely, since relief of the obstruction by a biliary fistula results in a fall in the serum phosphatase, although no bile has entered the intestine. Also return of the fistula bile to the gastrointestinal tract does not alter the rate at which the serum alkaline phosphatase decreases. Sherlock and Walshe (26) also claim that intravenous injection of calcium in subjects with obstructive jaundice does not reduce the serum alkaline phosphatase.

4. The sera of patients or animals with biliary obstruction contain an 'activator' which increases the activity of alkaline phosphatase already present (Thannhauser *et al.* (27)). It is claimed that the serum from a dog with biliary obstruction or the serum from a patient with a high serum phosphatase, when added to normal serum, increases its alkaline phosphatase activity. Evidence interpreted as favoring the 'activator' theory was also reported by Cantarow (7). On the other hand, Albers (1) could find no evidence of an 'activator', nor could Delory and King (10) in a series of experiments in which the hydrogen ion concentration was carefully controlled.

If the increased alkaline phosphatase that occurs in biliary obstruction were due to liberation of the enzyme from white cells, one would not expect an 'activation' in experiments, in which serum only was used, such as those

reported by Thannhauser *et al.* (27), and Delory and King (10), but one would expect an increase in the alkaline phosphatase of the plasma after the intravenous administration of bile salt. There is an increase in the acid phosphatase of the plasma which occurs after an injection far too small to produce an increase in the alkaline phosphatase. It is concluded, therefore, that the retention of bile salt does not explain the increase in serum alkaline phosphatase of biliary obstruction. These experiments lend no support to the 'activator' theory of Thannhauser *et al.* (27), even in a modified form.

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ALKALINE AND ACID PHOSPHATASE IN CEREBROSPINAL FLUID

DATA FOR NORMAL FLUIDS AND FLUIDS FROM PATIENTS WITH MENINGITIS, POLIOMYELITIS, OR SYPHILIS[1]

By K. G. Colling and R. J. Rossiter

Abstract

Many normal cerebrospinal fluids contain an alkaline (pH 9.8) and an acid (pH 4.9) phosphatase. Both the alkaline and the acid phosphatase were significantly increased in the spinal fluids from patients with meningitis or poliomyelitis, but not in the fluids from patients with syphilis. The alkaline phosphatase activity was correlated with both the concentration of protein in the spinal fluid and with the white cell count, whereas the acid phosphatase was correlated with neither. When correction was made for the significant correlation between cell count and protein concentration, the partial correlation between alkaline phosphatase activity and both protein concentration and cell count remained significant statistically. In pathological conditions it appears likely that the alkaline phosphatase is derived partly from the polymorphonuclear leucocytes in the fluid and partly from the blood plasma. The acid phosphatase is probably derived from the lymphocytes of the fluid and possibly also from the blood plasma. It is unlikely that either of these enzymes comes from the substance of the brain or spinal cord. Acid phosphatase would be of more value than alkaline phosphatase as a diagnostic aid, since normal fluids contain much less of this enzyme.

Introduction

Previous publications from this laboratory have been concerned with the enzymes of white blood cells (6, 7, 18, 30, 31). Since pathological cerebrospinal fluids seemed a possible source of human white cells, a systematic study of the enzymes of spinal fluids from normal subjects and from patients with a number of diseases has been undertaken. Colling and Rossiter (5) have already reported that the true cholinesterase, but not the pseudocholinesterase, was significantly increased in the spinal fluids of patients with syphilis, while the pseudocholinesterase, and not the true cholinesterase, was increased in the fluids of patients with either meningitis or poliomyelitis. The true cholinesterase activity was correlated neither with the protein concentration nor with the cell count, while the pseudocholinesterase was correlated with the protein concentration and not with the cell count.

This paper concerns the phosphatases of normal and pathological cerebrospinal fluids. There are at least two phosphatases, or perhaps more correctly, phosphomonoesterases, in mammalian tissues (14, 29), an alkaline phosphatase with a pH optimum between 8.5–10, depending upon both the nature (26) and the concentration (6, 13, 20) of the substrate, and an acid phosphatase with a pH optimum between 4.5–5. Kaplan, Cohn, Levinson, and Stern (23), Fleischhacker (11), Kovács (28a), and Albers (1), all using a modification of the method of Bodansky (4), have reported the presence of a

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low concentration of alkaline phosphatase in normal cerebrospinal fluid. In certain pathological conditions the cerebrospinal alkaline phosphatase was increased. As far as we are aware there are no reports in the literature on the concentration of acid phosphatase in either normal or pathological cerebrospinal fluids.

Methods

Phosphatase was measured by a modification of the method of King and Armstrong (25) in which the phenol, liberated from the hydrolysis of disodium phenyl phosphate, is determined by the method of Folin and Ciocalteu (12). Carbonate–bicarbonate buffer pH 9.8 (27) was used for the determination of the alkaline phosphatase and citrate buffer pH 4.9 (35) was used for the acid phosphatase. Because magnesium ions greatly activate many phosphatases (2, 6, 9, 13, 19, 21), the final concentration of magnesium in the reaction mixture was 0.001 *M*. This concentration of magnesium is optimal for many phosphatases (21).

To a 15 ml. centrifuge tube was added:—

2 ml. buffer (either pH 9.8 or pH 4.9)
2 ml. 0.01 *M* disodium phenyl phosphate
0.5 ml. 0.01 *M* magnesium chloride
0.2 ml. cerebrospinal fluid
0.3 ml. distilled water.

The tube was stoppered and incubated for 18 hr. in a 37° C. water bath. At the end of this time 1.8 ml. Folin and Ciocalteu reagent (diluted 1 : 3) was added and, after mixing, the tube was centrifuged. The phenol in 4 ml. of the supernatant was determined as described by King and Armstrong (25). A blank tube was also set up in which the substrate, buffer, and magnesium were incubated alone and the cerebrospinal fluid was added just before the Folin and Ciocalteu phenol reagent. The color of the test was read against that of the blank in a Coleman Universal Spectrophotometer at 650 mμ. The results were recorded as the quantity of phenol (in μgm.) liberated by 100 ml. cerebrospinal fluid in one hour. The figures for alkaline phosphatase can be approximately converted into the more familiar King–Armstrong units per 100 ml. (27) by dividing by 4000. Those for the acid phosphatase can be approximately converted into King–Armstrong units per 100 ml. (35) by dividing by 1000.

There is a theoretical objection to using such a long incubation period. The activity of the enzyme was not strictly linear with time for 18 hr. Since this lengthy incubation was necessary if measurable optical densities were to be obtained, some compromise seemed desirable. Dilution tests have shown that the enzyme activity, measured under the conditions of the experiment, was proportional to the concentration of the enzyme. Other workers on cerebrospinal fluid phosphatase have used incubation periods of as long as 24 hr. (23).

A drop of toluol was added to the total volume of cerebrospinal fluid immediately after it was collected. Both test and blank were always run in duplicate.

Results

Normal Cerebrospinal Fluids

Table I shows the mean alkaline phosphatase activity of normal cerebrospinal fluids. These fluids were taken from apparently healthy adults under 55 years of age. The criteria of normality were the same as those described

TABLE I

ALKALINE PHOSPHATASE OF CEREBROSPINAL FLUID (μGM. PHENOL 100 ML. HR.)

Diagnosis	No. patients	Mean	S. E. mean	*P*
Normal	20	38	± 13	
Meningitis	13	608	±158	<0.01
Poliomyelitis	24	98	±177	<0.01
Syphilis, negative C. S. F. Wassermann	18	33	± 10	>0.7
Syphilis, positive C. S. F. Wassermann	11	73	± 35	>0.2

in our previous report (5). There was no history of systemic disease such as syphilis, tuberculosis, or diabetes. Each fluid had a normal protein concentration, cell count, and negative Wassermann and colloidal gold reactions. Many of the fluids were withdrawn from the subjects before the injection of a spinal anesthetic for a "cold" surgical operation. The presence of an alkaline phosphatase in some normal cerebrospinal fluids confirms the reports of other workers (1, 11, 23), but Table II shows that in 11 of the 20 normal fluids

TABLE II

DISTRIBUTION OF ALKALINE PHOSPHATASE IN CEREBROSPINAL FLUIDS

Diagnosis	Total No.	No. with alkaline phosphatase activity (μgm. phenol/100ml./hr.) between:					
		0	1–50	51–100	101–150	151–200	200
Normal	20	11	5	1	1	2	
Meningitis	13			1	1	1	10
Poliomyelitis	24	3	3	9	5	2	2
Syphilis, negative C.S.F. Wassermann	18	9	3	4	2		
Syphilis, positive C.S.F. Wassermann	11	5	3	0	1		2

studied there was no hydrolysis of disodium phenyl phosphate at pH 9.8. In three out of these 20 fluids the alkaline phosphatase activity exceeded twice the standard deviation (Table III).

TABLE III

ALKALINE PHOSPHATASE OF CEREBROSPINAL FLUID AND CLINICAL DIAGNOSIS

Diagnosis	No. patients	No. with normal alkaline phosphatase activity	No. with increased alkaline phosphatase activity	Percentage with increased alkaline phosphatase activity	χ^2	P
Normal	20	17	3	15		
Meningitis	13	3	10	77	12.6	<0.01
Poliomyelitis	24	20	4	17	0.00	>0.9
Syphilis, negative C.S.F. Wassermann	18	18	0	0	0.00	>0.9
Syphilis, positive C.S.F. Wassermann	11	9	2	18	0.05	>0.7

Table IV shows the mean acid phosphatase activity of the normal fluids. By no means all of them hydrolyzed disodium phenyl phosphate at pH 4.9. Of the 20 normal fluids studied only six had a measurable acid phosphatase activity (Table VI).

Meningitis

Table I shows that the mean alkaline phosphatase activity of a group of fluids from patients with meningitis was much greater than that of the normal fluids. For the patients with meningitis there is a pronounced shift to the right in Table II, the alkaline phosphatase activity of 10 of the 13 fluids exceeding that of the most active normal fluid. The patients studied included two with meningococcal meningitis, three with pneumococcal meningitis, three with influenzal meningitis, and five with tuberculous meningitis. Table III shows that, of these 13 fluids, 10 had an increased alkaline phosphatase activity, i.e. an activity that exceeded the mean of the normal fluids by more than twice the standard deviation. A χ^2 test showed the increase in alkaline phosphatase activity of the meningitic fluids to be highly significant statistically ($P < 0.01$).

The mean acid phosphatase activity of the fluids from patients with meningitis was also much greater than that of the normal fluids (Table IV). The fluids included two from patients with meningococcal meningitis, two from patients with pneumococcal meningitis, three from patients with influenzal meningitis, and two from patients with tuberculous meningitis. There appeared to be no definite relation between the enzyme activity and the type

TABLE IV

ACID PHOSPHATASE OF CEREBROSPINAL FLUID (μGM. PHENOL/100 ML./HR.)

Diagnosis	No. patients	Mean	S. E. mean	*P*
Normal	20	5.5	± 2.4	
Meningitis	9	476	±323	<0.05
Poliomyelitis	24	71	± 25	<0.02
Syphilis, negative C.S.F. Wassermann	16	12.9	± 6.3	>0.2
Syphilis, positive C.S.F. Wassermann	11	12.5	± 7.4	>0.2

of cell predominating in the spinal fluid, although it was noticed that the acid phosphatase activity was usually high in the fluids from patients with tuberculous meningitis where the predominating type of cell was the lymphocyte.

The scatter of the acid phosphatase values for the meningitic fluids was so great that the difference between the mean acid phosphatase activity of the meningitic and normal fluids was just significant statistically ($P < 0.05$). This is brought out in Table V where it is seen that, of the nine fluids from patients with meningitis, two had no acid phosphatase activity whatsoever.

TABLE V

DISTRIBUTION OF ACID PHOSPHATASE IN CEREBROSPINAL FLUIDS

Diagnosis	Total No.	No. with acid phosphatase activity (μgm. phenol/100 ml./hr.) between:				
		0	1 – 25	26 – 50	51 – 75	>76
Normal	20	14	4	2		
Meningitis	9	2		2		5
Poliomyelitis	24	7	2	5	3	7
Syphilis, negative C.S.F. Wassermann	16	12		2	2	
Syphilis, positive C.S.F. Wassermann	11	8	1		2	

One of these was from a patient with pneumococcal meningitis and one from a patient with influenzal meningitis. Both had an increased alkaline phosphatase activity.

Table VI shows that, of the nine fluids on which the acid phosphatase activity was determined, seven exceeded the mean of the normal fluids by more than twice the standard deviation. A χ^2 test showed this increase in acid phosphatase activity to be significant statistically ($P < 0.01$).

TABLE VI

ACID PHOSPHATASE OF CEREBROSPINAL FLUID AND CLINICAL DIAGNOSIS

Diagnosis	No. patients	No. with normal acid phosphatase activity	No. with increased acid phosphatase activity	Percentage with increased acid phosphatase activity	χ^2	P
Normal	20	18	2	10		
Meningitis	9	2	7	78	13.3	<0.01
Poliomyelitis	24	9	15	63	12.7	<0.01
Syphilis, negative C.S.F. Wassermann	16	12	4	25	1.4	>0.2
Syphilis, positive C.S.F. Wassermann	11	9	2	18	0.4	>0.5

In many of the meningitic patients repeated spinal fluid examinations were made for alkaline and acid phosphatase activity. These revealed a similar increased activity but, with clinical improvement and decrease in spinal fluid cell count and protein concentration, the phosphatase activity decreased. The findings for the first test only for each patient is reported in the tables.

Poliomyelitis

The following observations were made on the spinal fluids from a group of patients with established poliomyelitis contracted during a mild poliomyelitis epidemic in the autumn of 1948. Table I shows that the mean alkaline phosphatase activity of the spinal fluids from patients with symptomatic poliomyelitis was significantly greater than that of the mean of the normal group ($P < 0.01$). In Table II there is a distinct shift to the right for the fluids from patients with poliomyelitis, although the shift is not as pronounced as that observed for the fluids of those with meningitis. Because the scatter of the alkaline phosphatase activity of the control group was so great, only four of the 24 fluids from patients with poliomyelitis had an increased activity i.e. an activity which exceeded the mean of the control group by more than twice the standard deviation. A χ^2 test showed this not to be significantly different from the normal group.

The mean acid phosphatase activity of the spinal fluids from patients with poliomyelitis also was significantly ($P < 0.02$) greater than the mean of the control group (Table IV). In Table V there is a shift to the right for the fluids from patients with poliomyelitis. Of the 24 patients with poliomyelitis, 15 had an increased acid phosphatase activity (Table VI). A χ^2 test showed this to be highly significant statistically ($P < 0.01$). Of the nine fluids that failed to show an acid phosphatase activity greater than the mean of the normal group by more than twice the standard deviation, four were from patients who had passed the acute phase of their illness and who were detained

in hospital for physiotherapy. Thus, of the 20 patients with acute poliomyelitis, only five failed to show a significant increase in the spinal fluid acid phosphatase.

A group of seven undiagnosed patients were admitted to hospital during the poliomyelitis epidemic. These patients were suspected of having poliomyelitis. The clinical diagnosis ranged from 'influenza' to 'upper respiratory infection' and 'early subclinical poliomyelitis'. All had normal cerebrospinal fluid cell count and protein concentration. In the spinal fluids from five of these seven patients neither the alkaline nor the acid phosphatase activity was increased. Four of these five patients with normal spinal fluid phosphatase were subsequently discharged without developing poliomyelitis while the fifth developed acute rheumatic fever. There was a significant increase in both the alkaline and the acid phosphatase activity in the spinal fluid of the remaining two undiagnosed patients. On the basis of cerebrospinal fluid findings one of these was later diagnosed as having 'subclinical poliomyelitis' and the other subsequently developed a typical attack of acute symptomatic poliomyelitis.

Syphilis

Table I gives the alkaline phosphatase activity of a series of fluids from a number of patients with syphilis (positive blood Wassermann reaction). The fluids were divided into two groups according as to whether the spinal fluid Wassermann reaction was positive or negative. Both Table I and Table II show that there was no significant increase in the alkaline phosphatase activity of either group, while Tables IV and V show that the same was true for the activity of the acid phosphatase.

Protein Concentration

Table VII gives the coefficient of correlation between the concentration of protein in the spinal fluid and both the alkaline and the acid phosphatase

TABLE VII

CORRELATION BETWEEN ALKALINE AND ACID PHOSPHATASE ACTIVITY OF CEREBROSPINAL FLUIDS AND CONCENTRATION OF PROTEIN

Enzyme	Number of observations	Coefficient of correlation (r)	S.E. of r
Alkaline phosphatase	71	0.60	± 0.08
Acid phosphatase	69	0.11	± 0.12

activity. In compiling these statistics the fluids from all groups were pooled, regardless of clinical diagnosis. There was a statistically significant ($r = 0.60 \pm 0.08$) correlation between the alkaline phosphatase activity and protein concentration, but that between acid phosphatase activity and protein concentration was not significant ($r = 0.11 \pm 0.12$).

Cell Count

The alkaline phosphatase activity was also significantly ($r = 0.75 \pm 0.05$) correlated with the number of cells in the cerebrospinal fluid (Table VIII), whereas the acid phosphatase activity was not ($r = 0.03 \pm 0.12$).

TABLE VIII

CORRELATION BETWEEN ALKALINE AND ACID PHOSPHATASE ACTIVITY OF CEREBROSPINAL FLUIDS AND CELL COUNT

Enzyme	Number of observations	Coefficient of correlation (r)	S.E. of r
Alkaline phosphatase	71	0.75.	±0.05
Acid phosphatase	69	0.03	±0.12

Thus the alkaline phosphatase activity was correlated with both the protein concentration and the cell count, while the acid phosphatase activity was correlated with neither. Table IX shows that, although there also was a significant correlation between cell count and protein concentration ($r_{23} = 0.45 \pm 0.10$), the coefficient of partial correlation between alkaline phosphatase activity and protein concentration, with cell count excluded ($r_{12 \cdot 3} = 0.44 \pm 0.10$), was statistically significant. The same was true for the coefficient of

TABLE IX

CORRELATION COEFFICIENTS AND PARTIAL CORRELATION COEFFICIENTS BETWEEN ALKALINE PHOSPHATASE ACTIVITY, PROTEIN CONCENTRATION, AND CELL COUNT OF 71 CEREBROSPINAL FLUIDS

	Correlation between:	Coefficient of correlation (r)	S.E. of r
r_{12}	Alkaline phosphatase activity and protein concentration	0.60	±0.08
r_{13}	Alkaline phosphatase activity and cell count	0.75	±0.05
r_{23}	Protein concentration and cell count	0.45	±0.10
$r_{12 \cdot 3} = \frac{r_{12} - r_{13} \times r_{23}}{\sqrt{1 - r_{13}^2} \times \sqrt{1 - r_{23}^2}}$	Alkaline phosphatase activity and protein concentration (cell count excluded)	0.44	±0.10
$r_{13 \cdot 2} = \frac{r_{13} - r_{12} \times r_{23}}{\sqrt{1 - r_{12}^2} \times \sqrt{1 - r_{23}^2}}$	Alkaline phosphatase activity and cell count (protein concentration excluded)	0.68	±0.06

partial correlation between the alkaline phosphatase activity and cell count with protein concentration excluded ($r_{13\cdot2} = 0.68 \pm 0.06$).

Discussion

Many of the normal cerebrospinal fluids had both an alkaline and an acid phosphatase activity, but this finding was far from constant. A small percentage of the fluids had no phosphatase activity at all. The finding of an alkaline phosphatase in normal spinal fluid is a confirmation of the work of Kaplan *et al.* (23), Fleischhacker (11), Kovács (28a), and Albers (1).

It is well known that red blood cells contain an active acid phosphatase, sometimes of the order of 400 King–Armstrong units per 100 ml. packed cells (3, 28). A spinal fluid with 100 red cells per cu. mm. would thus have an acid phosphatase activity of approximately 4 μgm. phenol per 100 ml. It is possible that contamination with blood at the time of lumbar puncture could account for a slight acid phosphatase activity in some of the fluids, although the low (5.5 ± 2.4 μgm. phenol per 100 ml.) acid phosphatase activity of normal fluids would suggest that such contamination, if it occurs, is not great. All fluids obviously contaminated with blood were discarded. It is therefore extremely unlikely that the increase in acid phosphatase activity found in the spinal fluids of patients with meningitis or poliomyelitis can be explained in terms of red cell contamination.

The literature on the phosphatase activity of spinal fluids from patients with disease is not extensive. Kaplan *et al.* (23) reported an increase in the alkaline phosphatase activity of fluids from patients with purulent meningitis and a lesser increase in the fluids from patients with tuberculous meningitis, a result confirmed by Albers (1). Kaplan *et al.* (23) also reported a slight increase in the fluids from patients with poliomyelitis. Fleischhacker (11) found an increase in alkaline phosphatase activity in the fluids of 20 out of the 25 patients studied with neurosyphilis. Since he reported no figures and gave no definition of what he considered 'an increase' in alkaline phosphatase activity, it is possible that his figures were not significantly different from ours. Kovács (28a) also found that the alkaline phosphatase of the spinal fluid from patients with a number of diseases was increased.

As has been pointed out previously (5), Kaplan *et al.* (24) suggested that enzymes in normal cerebrospinal fluid may be derived from either the tissue of the central nervous system, i.e. brain or spinal cord, or from the blood plasma. They also suggested that the enzyme activity of the fluid from patients with disease could be increased by one or more of the following methods:—

(a) An increase in enzyme-containing cells in the cerebrospinal fluid.

(b) An increase in the permeability of the membrane between the blood plasma and the cerebrospinal fluid, with the passage of plasma enzymes into the cerebrospinal fluid.

(c) A destruction of the tissue of the brain or spinal cord with the appearance of central nervous system enzymes in the cerebrospinal fluid.

Alkaline Phosphatase

The finding that the alkaline phosphatase activity was correlated significantly with cell count, even when the protein concentration was excluded, suggests that this enzyme may be present in the white cells. This is to be expected when it is remembered that the white cells of both the rabbit (6, 7) and man (18) have a high alkaline phosphatase activity. This is sufficient to explain the increased alkaline phosphatase activity of the spinal fluids from patients with purulent meningitis observed by Kaplan *et al.* (23) and confirmed in our study. These workers also reported that the alkaline phosphatase activity of fluids from patients with purulent meningitis was greater than that of fluids from patients with tuberculous meningitis. This can be explained by the observation of Haight and Rossiter (18) that the polymorphonuclear leucocyte is rich in alkaline phosphatase, whereas the lymphocyte contains little of this enzyme.

That there was a slight, but very variable, increase in the alkaline phosphatase of spinal fluids from patients with poliomyelitis is probably explained by the variability of the type of cell commonly found in the cerebrospinal fluids of patients with this disease. Early in the disease, when polymorphonuclear leucocytes predominate, one would expect an increase in alkaline phosphatase activity, but later, when lymphocytes exceed polymorphonuclear leucocytes, one would expect this increase in alkaline phosphatase activity to disappear.

Unfortunately differential cell counts were not done because, at the time, the great importance of the type of cell was not realized. It seems clear that most of the increase in alkaline phosphatase activity of pathological fluids is brought about by Method (*a*), the type of cell responsible being the polymorphonuclear leucocyte, which is rich in this enzyme. A similar conclusion was reached by Kaplan *et al.* (24).

Normal cerebrospinal fluids may possess an alkaline phosphatase activity and yet contain no cells. Since the blood plasma is rich in alkaline phosphatase (14, 29), it is possible that some of this enzyme passes through the membrane between the blood plasma and the cerebrospinal fluid. Besides being correlated with the white cell count, the alkaline phosphatase activity was also significantly correlated with the concentration of protein in the spinal fluid, even when the cell count is excluded. In this respect it is similar to pseudocholinesterase. Each of these two enzymes is increased in the spinal fluids from patients with either meningitis or poliomyelitis and the concentration of each is significantly correlated with the concentration of protein in the fluid. Since pseudocholinesterase is present in the blood plasma and absent from both the white cells and the substance of the central nervous system ((5) for references), it is likely that this enzyme reaches the spinal fluid from the plasma. Such a suggestion has been made by Tower and McEachern (33, 34). Alkaline phosphatase may also reach the spinal fluid from the plasma in the same fashion. If this were so, the increased alkaline phosphatase of pathological spinal fluids, besides occurring by Method (*a*),

could also occur by Method (*b*). This was also suggested by Kaplan *et al.* (24) to explain the observation that, after removing the cells containing alkaline phosphatase by centrifuging, some alkaline phosphatase activity still remained. Method (*b*) might also explain the existence of alkaline phosphatase activity in normal spinal fluids containing no cells. Tower and McEachern (33) reported that in spinal fluids from patients with craniocerebral trauma the pseudocholinesterase activity was increased. It would be interesting to know if there was also an increase in alkaline phosphatase in the fluids of patients with this condition.

Tissue of the central nervous system has been shown to contain an alkaline phosphatase by chemical methods (8, 10, 15) and histochemically (22) by the technique of Gomori (16) and Takamatsu (32). Tissue of the nervous system also contains a true cholinesterase, which was increased in the spinal fluids of patients with syphilis, a disease that produces evident damage in the central nervous system, but not in fluids from patients with meningitis or poliomyelitis (5). It was suggested that the increased amounts of this enzyme in the spinal fluids of patients with syphilis came from the substance of the nervous system. Unlike true cholinesterase, there was no significant increase in the alkaline phosphatase activity of fluids from patients with syphilis, although there was an increase in fluids from patients with meningitis or poliomyelitis. It would thus seem unlikely that the increase in alkaline phosphatase activity in the fluids of patients with these diseases is brought about by Method (*c*).

Acid Phosphatase

For both man and the rabbit the concentration of acid phosphatase is high in the lymphocyte and low in the polymorphonuclear leucocyte. Also for man the activity of the acid phosphatase in the lymphocyte is approximately eight times that of the alkaline phosphatase in the polymorphonuclear leucocyte (18). One would expect, therefore, an increase in acid phosphatase activity in conditions where there is an increase in lymphocytes in the cerebrospinal fluid. This increase should be all the more pronounced since the activity of the acid phosphatase of normal fluids is very much less than that of the alkaline phosphatase. In conditions where there is an increase in the number of lymphocytes in the spinal fluid (e.g. meningitis and poliomyelitis) there was a very significant increase in acid phosphatase activity. The activity of the acid phosphatase, unlike that of the alkaline phosphatase, was not correlated significantly with the total cell count, but this is to be expected since in most instances the majority of the cells were polymorphonuclear leucocytes. The acid phosphatase was present in a few cells at a high concentration, whereas the alkaline phosphatase was present in many cells at a lower concentration. It seems likely that most of the acid phosphatase in pathological fluids is accounted for by Method (*a*).

Acid phosphatase is found in quite high concentration in blood plasma (14, 29). Also the acid phosphatase was increased in fluids from patients

with meningitis or poliomyelitis, conditions in which there was also an increase in the spinal fluid alkaline phosphatase and pseudocholinesterase, two enzymes part or all of which, it has been suggested, comes from the blood plasma. Since the acid phosphatase activity, unlike that of the alkaline phosphatase or pseudocholinesterase, is not correlated with the protein concentration, Method (*b*) would appear to be of less importance for acid phosphatase. The method cannot be excluded, however, and may be the chief source of acid phosphatase in those normal fluids that have enzyme activity.

Acid phosphatase has also been demonstrated in the substance of the central nervous system by chemical methods (8, 10, 15) and histochemically (36) by the method of Gomori (17) but, for the same reasons advanced for alkaline phosphatase, it is considered unlikely that the acid phosphatase is increased by Method (*c*).

From the point of view of diagnosis, cerebrospinal fluid acid phosphatase would appear to be of value in poliomyelitis. Because of the wide scatter in the control group, satisfactory conclusions cannot be drawn from alkaline phosphatase estimations. Both alkaline and acid phosphatase determinations could be used as an aid in the diagnosis of meningitis. However these tests have no obvious advantages over the established diagnostic procedures for this disease. The test would appear to be of no value in the diagnosis of syphilis of the nervous system.

This work must be considered as preliminary, and of an exploratory nature only. Before phosphatase estimations could be recommended as a routine diagnostic procedure a full investigation of spinal fluids from patients with diseases of the central nervous system other than those already investigated would have to be undertaken. In addition a careful study of the changes of the phosphatase activity of the spinal fluid from the same patient throughout the entire course of such diseases would be highly desirable.

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